OSTEOPROTEGERIN BINDING PROTEINS AND RECEPTORS

This application is a continuation of application Serial No. 09/721,212, filed November 21,
5   2000, which is a continuation of application Serial No. 09/052,521 filed March 30, 1998, now U.S. Patent No. 6,316,408, which is a continuation-in-part of application Serial No. 08/880,855 filed June 23, 1997, abandoned, which is a continuation-in-part of
10 application Serial No. 08/842,842, filed April 16, 1997, now U.S. Patent No. 5,843,678, all of which are hereby incorporated by reference .--

Field of the Invention
15   The present invention relates to polypeptides which are involved in osteoclast differentiation. More particularly, the invention relates to osteoprotegerin binding proteins, nucleic acids encoding the proteins, expression vectors and host cells for production of the
20 proteins, and binding assays. Compositions and methods for the treatment of bone diseases, such as osteoporosis, bone loss from arthritis, Paget's disease, and hypercalcemia, are also described.
            The invention also relates to receptors for
25 osteoprotegerin binding proteins and methods and compositions for the treatment of bone diseases using the receptors.

Background of the Invention
30   Living bone tissue exhibits a dynamic equilibrium between deposition and resorption of bone. These processes are mediated primarily by two cell types: osteoblasts, which secrete molecules that comprise the organic matrix of bone; and osteoclasts,
35 which promote dissolution of the bone matrix and

solubilization of bone salts. In young individuals
with growing bone, the rate of bone deposition exceeds
the rate of bone resorption, while in older individuals
the rate of resorption can exceed deposition. In the
5   latter situation, the increased breakdown of bone leads
to reduced bone mass and strength, increased risk of
fractures, and slow or incomplete repair of broken
bones.

Osteoclasts are large phagocytic
10   multinucleated cells which are formed from
hematopoietic precursor cells in the bone marrow.
Although the growth and formation of mature functional
osteoclasts is not well understood, it is thought that
osteoclasts mature along the monocyte/macrophage cell
15   lineage in response to exposure to various growth-
promoting factors. Early development of bone marrow
precursor cells to preosteoclasts are believed to
mediated by soluble factors such as tumor necrosis
factor-α (TNF-α), tumor necrosis factor-β (TNF-β),
20   interleukin-1 (IL-1), interleukin-4 (IL-4),
interleukin-6 (IL-6), and leukemia inhibitory factor
(LIF). In culture, preosteoclasts are formed in the
presence of added macrophage colony stimulating factor
(M-CSF). These factors act primarily in early steps of
25   osteoclast development. The involvement of polypeptide
factors in terminal stages of osteoclast formation has
not been extensively reported. It has been reported,
however, that parathyroid hormone stimulates the
formation and activity of osteoclasts and that
30   calcitonin has the opposite effect, although to a
lesser extent.

Recently, a new polypeptide factor, termed
osteoprotegerin (OPG), has been described which
negatively regulated formation of osteoclasts in vitro
35   and in vivo (see co-owned and co-pending U.S. Serial

Nos. 08/577,788 filed December 22, 1995, 08/706,945
filed September 3, 1996, and 08/771,777, filed December
20, 1996, hereby incorporated by reference; and PCT
Application No. WO96/26271). OPG dramatically

5   increased the bone density in transgenic mice
expressing the OPG polypeptide and reduced the extent
of bone loss when administered to ovariectomized rats.
An analysis of OPG activity in in vitro osteoclast
formation revealed that OPG does not interfere with the

10   growth and differentiation of monocyte/macrophage
precursors, but more likely blocks the differentiation
of osteoclasts from monocyte/macrophage precursors.
Thus OPG appears to have specificity in regulating the
extent of osteoclast formation.

15   OPG comprises two polypeptide domains having
different structural and functional properties. The
amino-terminal domain spanning about residues 22-194 of
the full-length polypeptide (the N-terminal methionine
is designated residue 1) shows homology to other

20   members of the tumor necrosis factor receptor (TNFR)
family, especially TNFR-2, through conservation of
cysteine rich domains characteristic of TNFR family
members. The carboxy terminal domain spanning residues
194-401 has no significant homology to any known

25   sequences. Unlike a number of other TNFR family
members, OPG appears to be exclusively a secreted
protein and does not appear to be synthesized as a
membrane associated form.

         Based upon its activity as a negative

30   regulator of osteoclast formation, it is postulated
that OPG may bind to a polypeptide factor involved in
osteoclast differentiation and thereby block one or
more terminal steps leading to formation of a mature
osteoclast.

It is therefore an object of the invention to identify polypeptides which interact with OPG. Said polypeptides may play a role in osteoclast maturation and may be useful in the treatment of bone diseases.

Summary of the Invention

A novel member of the tumor necrosis factor family has been identified from a murine cDNA library expressed in COS cells screened using a recombinant OPG-Fc fusion protein as an affinity probe. The new polypeptide is a transmembrane OPG binding protein which is predicted to be 316 amino acids in length, and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. OPG binding proteins of the invention may be membrane-associated or may be in soluble form.

The invention provides for nucleic acids encoding an OPG binding protein, vectors and host cells expressing the polypeptide, and method for producing recombinant OPG binding protein. Antibodies or fragments thereof which specifically bind OPG binding protein are also provided.

OPG binding proteins may be used in assays to quantitate OPG levels in biological samples, identify cells and tissues that display OPG binding protein, and identify new OPG and OPG binding protein family members. Methods of identifying compounds which interact with OPG binding protein are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of OPG binding protein activity.

OPG binding proteins are involved in osteoclast differentiation and the level of osteoclast

activity in turn modulates bone resorption. OPG binding protein agonists and antagonists modulate osteoclast formation and bone resorption and may be used to treat bone diseases characterized by changes in

5   bone resorption, such as osteoporosis, hypercalcemia, bone loss due to arthritis metastasis, immobilization or periodontal disease, Paget's disease, osteopetrosis, prosthetic loosening and the like. Pharmaceutical compositions comprising OPG binding proteins and OPG

10   binding protein agonists and antagonists are also encompassed by the invention.

Receptors for OPG binding proteins have also been identified from a marine cDNA library constructed from bone marrow cells which bind to a fluorescent-

15   label OPG binding protein. The receptors may be used to identify agonists and antagonists of OPG binding protein interactions with its receptor which may be used to treat bone disease.

20   Description of the Figures

Figure 1. (SEQ ID NOS: 1 and 2) Structure and sequence of the 32D-F3 insert encoding OPG binding protein. Predicted transmembrane domain and sites for asparagine-linked carbohydrate chains are underlined.

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Figure 2. OPG binding protein expression in COS-7 cells transfected with pcDNA/32D-F3. Cells were lipofected with pcDNA/32D-F3 DNA, the assayed for binding to either goat anti-human IgG1 alkaline

30   phosphatase conjugate (secondary alone), human OPG[22-201]-Fc plus secondary (OPG-Fc), or a chimeric ATAR extracellular domain-Fc fusion protein (sATAR-Fc). ATAR is a new member of the TNFR superfamily, and the sATAR-Fc fusion protein serves as a control for both

human IgG1 Fc domain binding, and generic TNFR releated
protein, binding to 32D cell surface molecules.

Figure 3. Expression of OPG binding protein
in human tissues. Northern blot analysis of human
tissue mRNA (Clontech) using a radiolabeled 32D-F3
derived hybridization probe. Relative molecular mass
is indicated at the left in kilobase pairs (kb).
Arrowhead on right side indicates the migration of an
approximately 2.5 kb transcript detected in lymph node
mRNA. A very faint band of the same mass is also
detected in fetal liver.

Figure 4. (SEQ ID NOS: 3 and 4) Structure
and sequence of the pcDNA/ hu OPGbp 1.1 insert encoding
the human OPG binding protein. The predicted
transmembrane domain and site for asparagine-linked
carbohydrate chains are underlined.

Figure 5. Stimulation of osteoclast
development in vitro from bone marrow macrophage and
ST2 cell cocultures treated with recombinant murine OPG
binding protein [158-316]. Cultures were treated with
varying concentrations of murine OPG binding protein
ranging from 1.6 to 500 ng/ml. After 8-10 days,
cultures were lysed, and TRAP activity was measured by
solution assay. In addition, some cultures were
simultaneously treated with 1, 10, 100, 500, and 1000
ng/ml of recombinant murine OPG [22-401]-Fc protein.
Murine OPG binding protein induces a dose-dependent
stimulation in osteoclast formation, whereas OPG [22-
401]-Fc inhibits osteoclast formation.

Figure 6. Stimulation of osteoclast
development from bone marrow precursors in vitro in the

presence of M-CSF and murine OPG binding protein [158-316]. Mouse bone marrow was harvested, and cultured in the presence 250, 500, 1000, and 2000 U/ml of M-CSF. Varying concentrations of OPG binding protein [158-316], ranging from 1.6 to 500 ng/ml, were added to these same cultures. Osteoclast development was measured by TRAP solution assay.

Figure 7. Osteoclasts derived from bone marrow cells in the presence of both M-CSF and OPG binding protein [158-316] resorb bone in vitro. Bone marrow cells treated with either M-CSF, OPG binding protein, or with both factors combined, were plated onto bone slices in culture wells, and were allowed to develop into mature osteoclasts. The resulting cultures were then stained with Toluidine Blue (left column), or histochemically to detect TRAP enzyme activity (right column). In cultures receiving both factors, mature osteoclasts were formed that were capable of eroding bone as judged by the presence of blue stained pits on the bone surface. This correlated with the presence of multiple large, multinucleated, TRAP positive cells.

Figure 8. Graph showing the whole blood ionized calcium (iCa) levels from mice injected with OPG binding protein, 51 hours after the first injection, and in mice also receiving concurrent OPG administration. OPG binding protein significantly and dose dependently increased iCa levels. OPG (1mg/kg/day) completely blocked the increase in iCa at a dose of OPG binding protein of 5ug/day, and partially blocked the increase at a dose of OPG binding protein of 25ug/day. (*), different to vehicle treated control (p < 0.05). (#), OPG treated iCa level significantly

different to level in mice receiving that dose of OPG
binding protein alone (p < 0.05).

Figure 9. Radiographs of the left femur and
tibia in mice treated with 0, 5, 25 or 100μg/day of OPG
binding protein for 3.5 days. There is a dose dependent
decrease in bone density evident most clearly in the
proximal tibial metaphysis of these mice, and that is
profound at a dose of 100μg/day.

Figure 10. (SEQ ID NOS: 42 and 43) Murine
ODAR cDNA sequence and protein sequence. Nucleic acid
sequence of the ~2.1 kb cDNA clone is shown, and
translation of the 625 residue long open reading frame
indicated above. The hydrophobic signal peptide is
underlined, and the hydrophobic transmembrane sequence
(residues 214-234) is in bold. Cysteine residues that
comprise the cysteine-rich repeat motifs in the
extracellular domain are in bold.

Figure 11. Immunofluorescent staining of
ODAR-Fc binding to OPG binding protein transfected
cells. COS-7 cells transfected with OPG binding
protein expression plasmid were incubated with human
IgG Fc (top panel), ODAR-Fc (middle panel) or OPG-Fc
(bottom panel). A FITC-labeled goat anti-human IgG Fc
antibody was used as a secondary antibody. Positive
binding cells were examined by confocal microscopy.

Figure 12 . Effects of ODAR-Fc on the
generation of osteoclasts from mouse bone marrow in
vitro. Murine bone marrow cultures were established as
in Example 8 and exposed to OPG binding protein (5
ng/ml) and CSF-1 (30 ng/ml). Various concentrations of
ODAR-Fc ranging from 1500 ng/ml to 65 ng/ml were added.

Osteoclast formation was assessed by TRAP cytochemistry
and the TRAP solution assay after 5 days in culture.

Figure 13. Bone mineral density in mice
after treatment for four days with ODAR-Fc at varying
doses. Mice received ODAR-Fc by daily subcutaneous
injection in a phosphate buffered saline vehicle.
Mineral density was determined from bones fixed in 70%
ETOH at the proximal tibial metaphysis mice by
peripheral quantitative computed tomography (pQCT)
(XCT-960M, Norland Medical Systems, Ft Atkinson, WI).
Two 0.5 mm cross-sections of bone, 1.5 mm and 2.0 mm
from the proximal end of the tibia were analyzed (XMICE
5.2, Stratec, Germany) to determine total bone mineral
density in the metaphysis. A soft tissue separation
threshold of 1500 was used to define the boundary of
the metaphyseal bone. ODAR-Fc produced a significant
increase in bone mineral density in the proximal tibial
metaphysis in a dose dependent manner. Group n = 4.

Detailed Description of the Invention
The invention provides for a polypeptide
referred to as an OPG binding protein, which
specifically binds OPG and is involved in osteoclast
differentiation. A cDNA clone encoding the murine form
of the polypeptide was identified from a library
prepared from a mouse myelomonocytic cell line 32-D and
transfected into COS cells. Transfectants were
screened for their ability to bind to an OPG[22-201]-Fc
fusion polypeptide (Example 1). The nucleic acid
sequence revealed that OPG binding protein is a novel
member of the TNF family and is most closely related to
AGP-1, a polypeptide previously described in co-owned
and co-pending U.S. Serial No. 08/660,562, filed June
7, 1996. (A polypeptide identical to AGP-1 and

designated TRAIL is described in Wiley et al. Immunity
3, 673-682 (1995)). OPG binding protein is predicted
to be a type II transmembrane protein having a
cytoplamsic domain at the amino terminus, a
5   transmembrane domain, and a carboxy terminal
extracellular domain (Figure 1). The amino terminal
cytoplasmic domain spans about residues 1-48, the
transmembrane domain spans about residues 49-69 and the
extracellular domain spans about residues 70-316 as
10   shown in Figure 1 (SEQ ID NO: 2). The membrane-
associated protein specifically binds OPG (Figure 2).
Thus OPG binding protein and OPG share many
characteristics of a receptor-ligand pair although it
is possible that other naturally-occurring receptors
15   for OPG binding protein exist.

       A DNA clone encoding human OPG binding
protein was isolated from a lymph node cDNA library.
The human sequence (Figure 4) is homologous to the
murine sequence. Purified soluble murine OPG binding
20   protein stimulated osteoclast formation in vitro and
induced hypercalcemia and bone resorption in vivo.

       OPG binding protein refers to a polypeptide
having an amino acid sequence of mammalian OPG binding
protein, or a fragment, analog, or derivative thereof,
25   and having at least the activity of binding OPG. In
preferred embodiments, OPG binding protein is of murine
or human origin. In another embodiment, OPG binding
protein is a soluble protein having, in one form, an
isolated extracellular domain separate from cytoplasmic
30   and transmembrane domains. OPG binding protein is
involved in osteoclast differentiation and in the rate
and extent of bone resorption, and was found to
stimulate osteoclast formation and stimulate bone
resorption.

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Nucleic Acids

The invention provides for isolated nucleic acids encoding OPG binding proteins. As used herein, the term nucleic acid comprises cDNA, genomic DNA, wholly or partially synthetic DNA, and RNA. The nucleic acids of the invention are selected from the group consisting of:

a) the nucleic acids as shown in Figure 1 (SEQ ID NO: 1) and Figure 4 (SEQ ID NO: 3);

b) nucleic acids which hybridize to the polypeptide coding regions of the nucleic acids shown in Figure 1 (SEQ ID NO: 1) and Figure 4 (SEQ ID NO: 3); and remain hybridized to the nucleic acids under high stringency conditions; and

c) nucleic acids which are degenerate to the nucleic acids of (a) or (b).

Nucleic acid hybridizations typically involve a multi-step process comprising a first hybridization step to form nucleic acid duplexes from single strands followed by a second hybridization step carried out under more stringent conditions to selectively retain nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency, wherein "high stringency" conditions refers to conditions of temperature and salt which are about 12-20°C below the melting temperature (T_m) of a perfect hybrid of part or all of the complementary strands corresponding to Figure 1 (SEQ. ID. NO: 2) and Figure 4 (SEQ ID NO: 4). In one embodiment, "high stringency" conditions refer to conditions of about 65°C and not more than about 1M Na+. It is understood that salt concentration,

temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions for
5 hybridization of nucleic acids and calculations of T_m for nucleic acid hybrids are described in Sambrook et al. Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, New York (1989).

The nucleic acids of the invention may
10 hybridize to part or all of the polypeptide coding regions of OPG binding protein as shown in Figure 1 (SEQ ID NO: 2) and Figure 4 (SEQ ID NO: 4); and therefore may be truncations or extensions of the nucleic acid sequences shown therein. Truncated or
15 extended nucleic acids are encompassed by the invention provided that they retain at least the property of binding OPG. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids. In another embodiment, the nucleic acid will encode a
20 polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the OPG binding
25 protein coding regions. Noncoding sequences include regulatory regions involved in expression of OPG binding protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.
30 In preferred embodiments, the nucleic acids of the invention encode mouse or human OPG binding protein. Nucleic acids may encode a membrane bound form of OPG binding protein or soluble forms which lack a functional transmembrane region. The predicted
35 transmembrane region for murine OPG binding protein

includes amino acid residues 49-69 inclusive as shown in Figure 1 (SEQ. ID. NO: 2). The predicted transmembrane region for human OPG binding protein includes residues 49-69 as shown in Figure 4 (SEQ ID NO: 4). Substitutions which replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble OPG binding protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of OPG binding protein. Nucleic acids encoding amino acid residues 70-316 as shown in Figure 1 (SEQ ID NO: 1), or fragments and analogs thereof, encompass soluble OPG binding proteins.

Nucleic acids encoding truncated forms of soluble human OPG binding proteins are also included. Soluble forms include residues 69-317 as shown in Figure 4 (SEQ ID NO: 3) and truncations thereof. In one embodiment, N-terminal truncations generate polypeptides from residues, 70-317, 71-317, 72-317, and so forth. In another embodiment, nucleic acids encode soluble OPGbp comprising residues 69-317 and N-terminal truncations thereof up to OPGbp [158-317], or alternatively, up to OPGbp [166-317].

Plasmid phuOPGbp 1.1 in E. coli strain DH10 encoding human OPG binding protein was deposited with the American Type Culture Collection, Rockville, MD on June 13, 1997 (ATCC Accession No. 98457).

Nucleic acid sequences of the invention may be used for the detection of sequences encoding OPG binding protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related OPG binding protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of OPG binding

protein by anti-sense technology or _in vivo_ gene
expression. Development of transgenic animals
expressing OPG binding protein is useful for production
of the polypeptide and for the study of _in vivo_
5   biological activity.

Vectors and Host Cells
          The nucleic acids of the invention will be
linked with DNA sequences so as to express biologically
10   active OPG binding protein. Sequences required for
expression are known to those skilled in the art and
include promoters and enhancer sequences for initiation
of RNA synthesis, transcription termination sites,
ribosome binding sites for the initiation of protein
15   synthesis, and leader sequences for secretion.
Sequences directing expression and secretion of OPG
binding protein may be homologous, i.e., the sequences
are identical or similar to those sequences in the
genome involved in OPG binding protein expression and
20   secretion, or they may be heterologous. A variety of
plasmid vectors are available for expressing OPG
binding protein in host cells (see, for example,
Methods in Enzymology v. 185, Goeddel, D.V. ed.,
Academic Press (1990)). For expression in mammalian
25   host cells, a preferred embodiment is plasmid pDSRα
described in PCT Application No. 90/14363. For
expression in bacterial host cells, preferred
embodiments include plasmids harboring the lux promoter
(see co-owned and co-pending U.S. Serial No.
30   08/577,778, filed December 22, 1995). In addition,
vectors are available for the tissue-specific
expression of OPG binding protein in transgenic
animals. Retroviral and adenovirus-based gene transfer
vectors may also be used for the expression of OPG

binding protein in human cells for _in vivo_ therapy (see PCT Application No. 86/00922).

Procaryotic and eucaryotic host cells expressing OPG binding protein are also provided by the

5   invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. OPG binding protein may also be produced in transgenic animals such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate

10   host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences encoding OPG binding protein as shown in Figure 1 or a portion thereof, such as the extracellular domain or the cytoplasmic domain.

15   Nucleic acids encoding OPG binding proteins may be modified by substitution of codons which allow for optimal expression in a given host. At least some of the codons may be so-called preference codons which do not alter the amino acid sequence and are frequently

20   found in genes that are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for OPG binding protein expression include,

25   but are not limited to COS, CHOd-, 293 and 3T3 cells. A preferred bacterial host cell is _Escherichia coli_.

Polypeptides

The invention also provides OPG binding

30   protein as the product of procaryotic or eucaryotic expression of an exogenous DNA sequence, i.e., OPG binding protein is recombinant OPG binding protein. Exogenous DNA sequences include cDNA, genomic DNA and synthetic DNA sequences. OPG binding protein may be

35   the product of bacterial, yeast, plant, insect or

mammalian cells expression, or from cell-free
translation systems. OPG binding protein produced in
bacterial cells will have an N-terminal methionine
residue. The invention also provides for a process of
5   producing OPG binding protein comprising growing
procaryotic or eucaryotic host cells transformed or
transfected with nucleic acids encoding OPG binding
protein and isolating polypeptide expression products
of the nucleic acids.

10   Polypeptides which are mammalian OPG binding
proteins or are fragments, analogs or derivatives
thereof are encompassed by the invention. In a
preferred embodiment, the OPG binding protein is human
OPG binding protein. A fragment of OPG binding protein
15   refers to a polypeptide having a deletion of one or
more amino acids such that the resulting polypeptide
has at least the property of binding OPG. Said
fragments will have deletions originating from the
amino terminal end, the carboxy terminal end, and
20   internal regions of the polypeptide. Fragments of OPG
binding protein are at least about ten amino acids, at
least about 20 amino acids, or at least about 50 amino
acids in length. In preferred embodiments, OPG binding
protein will have a deletion of one or more amino acids
25   from the transmembrane region (amino acid residues 49-
69 as shown in Figure 1), or, alternatively, one or
more amino acids from the amino-terminus up to and/or
including the transmembrane region (amino acid residues
1-49 as shown in Figure 1). In another embodiment, OPG
30   binding protein is a soluble protein comprising, for
example, amino acid residues 69-316, or 70-316, or
N-terminal or C-terminal truncated forms thereof, which
retain OPG binding activity. OPG binding protein is
also a human soluble protein as shown in Figure 4
35   comprising residues 69-317 as shown in Figure 4 and N-

terminal truncated forms thereof, e.g., 70-517, 71-517, 71-317, 72-317 and so forth. In a preferred embodiment, the soluble human OPG binding protein comprising residues 69-317 and N-terminal truncation thereof up to OPGbp [158-317], or alternatively up to OPG [166-317].

An analog of an OPG binding protein refers to a polypeptide having a substitution or addition of one or more amino acids such that the resulting polypeptide has at least the property of binding OPG. Said analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble OPG binding proteins. Fragments or analogs may be naturally occurring, such as a polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue

Also included in the invention are derivatives of OPG binding protein which are polypeptides that have undergone post-translational modifications (e.g., addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of procaryotic host cell expression. In particular, chemically modified derivatives of OPG binding protein which provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with water soluble polymers, such as polyethylene glycol and derivatives thereof (see for

example U.S. Patent No. 4,179,337). The chemical
moieties for derivitization may be selected from water
soluble polymers such as polyethylene glycol, ethylene
glycol/propylene glycol copolymers,
5   carboxymethylcellulose, dextran, polyvinyl alcohol and
the like. The polypeptides may be modified at random
positions within the molecule, or at predetermined
positions within the molecule and may include one, two,
three or more attached chemical moieties. Polypeptides
10   may also be modified at pre-determined positions in the
polypeptide, such as at the amino terminus, or at a
selected lysine or arginine residue within the
polypeptide. Other chemical modifications provided
include a detectable label, such as an enzymatic,
15   fluorescent, isotopic or affinity label to allow for
detection and isolation of the protein.

        OPG binding protein chimeras comprising part
or all of an OPG binding protein amino acid sequence
fused to a heterologous amino acid sequence are also
20   included. The heterologous sequence may be any
sequence which allows the resulting fusion protein to
retain the at least the activity of binding OPG. In a
preferred embodiment, the carboxy terminal
extracellular domain of OPG binding protein is fused to
25   a heterologous sequence. Such sequences include
heterologous cytoplasmic domains that allow for
alternative intracellular signaling events, sequences
which promote oligomerization such as the Fc region of
IgG, enzyme sequences which provide a label for the
30   polypeptide, and sequences which provide affinity
probes, such as an antigen-antibody recognition.

        The polypeptides of the invention are
isolated and purified from tissues and cell lines which
express OPG binding protein, either extracted from
35   lysates or from conditioned growth medium, and from

transformed host cells expressing OPG binding protein.
OPG binding protein may be obtained from murine
myelomonocytic cell line 32-D (ATCC accession no. CRL-
11346). Human OPG binding protein, or nucleic acids
5   encoding same, may be isolated from human lymph node or
fetal liver tissue. Isolated OPG binding protein is
free from association with human proteins and other
cell constituents.

        A method for the purification of OPG binding
10 protein from natural sources (e.g. tissues and cell
lines which normally express OPG binding protein) and
from transfected host cells is also encompassed by the
invention. The purification process may employ one or
more standard protein purification steps in an
15 appropriate order to obtain purified protein. The
chromatography steps can include ion exchange, gel
filtration, hydrophobic interaction, reverse phase,
chromatofocusing, affinity chromatography employing an
anti-OPG binding protein antibody or biotin-
20 streptavidin affinity complex and the like.

Antibodies
        Antibodies specifically binding the
polypeptides of the invention are also encompassed by
25 the invention. The antibodies may be produced by
immunization with full-length OPG binding protein,
soluble forms of OPG binding protein, or a fragment
thereof. The antibodies of the invention may be
polyclonal or monoclonal, or may be recombinant
30 antibodies, such as chimeric antibodies wherein the
murine constant regions on light and heavy chains are
replaced by human sequences, or CDR-grafted antibodies
wherein only the complementary determining regions are
of murine origin. Antibodies of the invention may also
35 be human antibodies prepared, for example, by

immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). The antibodies are useful for detecting OPG binding protein in biological samples,

5 thereby allowing the identification of cells or tissues which produce the protein In addition, antibodies which bind to OPG binding protein and block interaction with other binding compounds may have therapeutic use in modulating osteoclast differentiation and bone

10 resorption.

Antibodies to the OPG binding protein may be useful in treatment of bone diseases such as, osteoporosis and Paget's disease. Antibodies can be tested for binding to the OPG binding protein in the

15 absence or presence of OPG and examined for their ability to inhibit ligand (OPG binding protein) mediated osteoclastogenesis and/or bone resorption. It is also anticipated that the peptides themselves may act as an antagonist of the ligand:receptor interaction

20 and inhibit ligand-mediated osteoclastogenesis, and peptides of the OPG binding protein will be explored for this purpose as well.

Compositions

25   The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the OPG binding protein of the invention together with a pharmaceutically acceptable diluent, carrier,

30 solubilizer, emulsifier, preservative and/or adjuvant. The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an OPG binding protein agonist or antagonist. The term "therapeutically effective amount" means an

35 amount which provides a therapeutic effect for a

specified condition and route of administration. The
composition may be in a liquid or lyophilized form and
comprises a diluent (Tris, acetate or phosphate
buffers) having various pH values and ionic strengths,
5   solubilizer such as Tween or Polysorbate, carriers such
as human serum albumin or gelatin, preservatives such
as thimerosal or benzyl alcohol, and antioxidants such
as ascorbic acid or sodium metabisulfite. Selection of
a particular composition will depend upon a number of
10   factors, including the condition being treated, the
route of administration and the pharmacokinetic
parameters desired. A more extensive survey of
component suitable for pharmaceutical compositions is
found in Remington's Pharmaceutical Sciences, 18th ed.
15   A.R. Gennaro, ed. Mack, Easton, PA (1980).

            In a preferred embodiment, compositions
comprising soluble OPG binding proteins are also
provided. Also encompassed are compositions comprising
soluble OPG binding protein modified with water soluble
20   polymers to increase solubility, stability, plasma
half-life and bioavailability. Compositions may also
comprise incorporation of soluble OPG binding protein
into liposomes, microemulsions, micelles or vesicles
for controlled delivery over an extended period of
25   time. Soluble OPG binding protein may be formulated
into microparticles suitable for pulmonary
administration.

            Compositions of the invention may be
administered by injection, either subcutaneous,
30   intravenous or intramuscular, or by oral, nasal,
pulmonary or rectal administration. The route of
administration eventually chosen will depend upon a
number of factors and may be ascertained by one skilled
in the art.

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the coding region of OPG binding protein and/or flanking regions to cells and tissues as part of an anti-sense therapy regimen.

Methods of Use

OPG binding proteins may be used in a variety of assays for detecting OPG and characterizing interactions with OPG. In general, the assay comprises incubating OPG binding protein with a biological sample containing OPG under conditions which permit binding to OPG to OPG binding protein, and measuring the extent of binding. OPG may be purified or present in mixtures, such as in body fluids or culture medium. Assays may be developed which are qualitative or quantitative, with the latter being useful for determining the binding parameters (affinity constants and kinetics) of OPG to OPG binding protein and for quantitating levels of biologically active OPG in mixtures. Assays may also be used to evaluate the binding of OPG to fragments, analogs and derivatives of OPG binding protein and to identify new OPG and OPG binding protein family members.

Binding of OPG to OPG binding protein may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of labeled OPG are incubated with OPG binding protein samples for a specified period of time followed by measurement of bound OPG by filtration,

electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time resolved fluorescence (HTRF, Packard) can also be

5   implemented. Binding is detected by labeling OPG or an anti-OPG antibody with radioactive isotopes (125I, 35S, 3H), fluorescent dyes (fluorescein), lanthanide (Eu3+) chelates or cryptates, orbipyridyl-ruthenium (Ru2+) complexes. It is understood that the choice of a

10   labeled probe will depend upon the detection system used. Alternatively, OPG may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6, myc) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies which have a

15   detectable label as described above.

In an alternative method, OPG binding protein may be assayed directly using polyclonal or monoclonal antibodies to OPG binding proteins in an immunoassay. Additional forms of OPG binding proteins containing

20   epitope tags as described above may be used in solution and immunoassays.

Methods for identifying compounds which interact with OPG binding protein are also encompassed by the invention. The method comprises incubating OPG

25   binding protein with a compound under conditions which permit binding of the compound to OPG binding protein, and measuring the extent of binding. The compound may be substantially purified or present in a crude mixture. Binding compounds may be nucleic acids,

30   proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The compounds may be further characterized by their ability to increase or decrease OPG binding protein activity in order to determine whether they act as an agonist or an

35   antagonist.

OPG binding proteins are also useful for identification of intracellular proteins which interact with the cytoplasmic domain by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an OPG binding protein fused to a yeast GAL4-DNA binding domain may be used as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate a intracellular signaling mechanism associated with OPG binding protein and provide intracellular targets for new drugs that modulate bone resorption.

OPG binding protein may be used to treat conditions characterized by excessive bone density. The most common condition is osteopetrosis in which a genetic defect results in elevated bone mass and is usually fatal in the first few years of life. Osteopetrosis is preferably treated by administration of soluble OPG binding protein.

The invention also encompasses modulators (agonists and antagonists) of OPG binding protein and the methods for obtaining them. An OPG binding protein modulator may either increase or decrease at least one activity associated with OPG binding protein, such as ability to bind OPG or some other interacting molecule or to regulate osteoclast maturation. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, which interacts with OPG binding protein to regulate its activity. Potential polypeptide antagonists include antibodies which react with either soluble or membrane-associated forms of OPG binding protein, and soluble forms of OPG binding protein which comprise part or all of the extracellular

domain of OPG binding protein. Molecules which regulate OPG binding protein expression typically include nucleic acids which are complementary to nucleic acids encoding OPG binding protein and which

5 act as anti-sense regulators of expression.

OPG binding protein is involved in controlling formation of mature osteoclasts, the primary cell type implicated in bone resorption. An increase in the rate of bone resorption (over that of

10 bone formation) can lead to various bone disorders collectively referred to as osteopenias, and include osteoporosis, osteomyelitis, hypercalcemia, osteopenia brought on by surgery or steroid administration, Paget's disease, osteonecrosis, bone loss due to

15 rheumatoid arthritis, periodontal bone loss, immobilization, prosthetic loosing and osteolytic metastasis. Conversely, a decrease in the rate of bone resorption can lead to osteopetrosis, a condition marked by excessive bone density. Agonists and

20 antagonists of OPG binding protein modulate osteoclast formation and may be administered to patients suffering from bone disorders. Agonists and antagonists of OPG binding protein used for the treatment of osteopenias may be administered alone or in combination with a

25 therapeutically effective amount of a bone growth promoting agent including bone morphogenic factors designated BMP-1 to BMP-12, transforming growth factor-β and TGF-β family members, fibroblast growth factors FGF-1 to FGF-10, interleukin-1 inhibitors, TNFα

30 inhibitors, parathyroid hormone, E series prostaglandins, bisphosphonates and bone-enhancing minerals such as fluoride and calcium. Antagonists of OPG binding proteins may be particularly useful in the treatment of osteopenia.

35

Receptors for Osteoprotegerin Binding Proteins

The invention also provides for receptors which interact with OPG binding proteins. More particularly, the invention provides for an osteoclast differentiation and activation receptor (ODAR). ODAR is a transmembrane polypeptide which shows highest degree of homology to CD40, a TNF receptor family member. The nucleic acid sequence of murine ODAR and encoded polypeptide is shown in Figure 10. The human homolog of murine ODAR may be readily isolated by hybridization screening of a human cDNA or genomic library with the nucleic acid sequence of Figure 10. Procedures for cloning human ODAR are similar to those described in Example 5 for cloning human OPG binding proteins. The human homolog of the polypeptide shown in Figure 10 has appeared in Anderson et al. (Nature 390, 175-179 (1997)) and is referred to therein as RANK. RANK is characterized as a type I transmembrane protein having homology to TNF receptor family members and is involved in dendritic cell function.

Evidence for the interaction of ODAR and OPG binding protein is shown in Example 13. A soluble form of ODAR (ODAR-Fc fusion protein) prevents osteoclast maturation in vitro (Figure 12) and increases bone density in normal mice after subcutaneous injection (Figure 13). The results are consistent with OPG binding protein interacting with and activating ODAR to promote osteoclast maturation.

Osteoclast development and the rate and extent of bone resorption are regulated by the interaction of OPG binding protein and ODAR. Compounds which decrease or block the interaction of OPG binding protein and ODAR are potential antagonists of OPG binding protein activity and may disrupt osteoclast development leading to decreased bone resorption.

Alternatively, compounds which increase the interaction of OPG binding protein and ODAR are potential agonists which promote osteoclast development and enhance bone resorption.

5   A variety of assays may be used to measure the interaction of OPG binding protein and ODAR <u>in vitro</u> using purified proteins. These assays may be used to screen compounds for their ability to increase or decrease the rate or extent of binding to ODAR by

10   OPG binding protein. In one type of assay, ODAR protein can be immobilized by attachment to the bottom of the wells of a microtiter plate. Radiolabeled OPG binding protein (for example, iodinated OPG binding protein) and the test compound(s) can then be added

15   either one at a time (in either order) or simultaneously to the wells. After incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the extent of binding to ODAR by OPG binding protein in the presence

20   of the test compound. Typically, the compound will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in evaluation of the results. An alternative to this method involves

25   reversing the "positions" of the proteins, *i.e.*, immobilizing OPG binding protein to the mictrotiter plate wells, incubating with the test compound and radiolabeled ODAR, and determining the extent of ODAR binding (see, for example, chapter 18 of *Current*

30   *Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, New York, NY [1995]).

         As an alternative to radiolabelling, OPG binding protein or ODAR may be conjugated to biotin and the presence of biotinylated protein can then be

35   detected using streptavidin linked to an enzyme, such

as horse radish peroxidase [HRP] or alkaline
phosphatase [AP], that can be detected
colorometrically, or by fluorescent tagging of
streptavidin. An antibody directed to OPG binding
protein or ODAR that is conjugated to biotin may also
be used and can be detected after incubation with
enzyme-linked streptavidin linked to AP or HRP

OPG binding protein and ODAR may also be
immobilized by attachment to agarose beads, acrylic
beads or other types of such inert substrates. The
substrate-protein complex can be placed in a solution
containing the complementary protein and the test
compound; after incubation, the beads can be
precipitated by centrifugation, and the amount of
binding between OPG binding protein and ODAR can be
assessed using the methods described above.
Alternatively, the substrate-protein complex can be
immobilized in a column and the test molecule and
complementary protein passed over the column.
Formation of a complex between OPG binding protein and
ODAR can then be assessed using any of the techniques
set forth above, *i.e.*, radiolabeling, antibody binding,
or the like.

Another type of <u>in vitro</u> assay that is useful
for identifying a compound which increases or decreases
formation of an ODAR/OPG binding protein complex is a
surface plasmon resonance detector system such as the
Biacore assay system (Pharmacia, Piscataway, NJ). The
Biacre system may be carried out using the
manufacturer's protocol. This assay essentially
involves covalent binding of either OPG binding protein
or ODAR to a dextran-coated sensor chip which is
located in a detector. The test compound and the other
complementary protein can then be injected into the
chamber containing the sensor chip either

simultaneously or sequentially and the amount of complementary protein that binds can be assessed based on the change in molecular mass which is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.

In some cases, it may be desirable to evaluate two or more test compounds together for use in increasing or decreasing formation of ODAR/OPG binding protein complex. In these cases, the assays set forth above can be readily modified by adding such additional test compound(s) either simultaneously with, or subsequently to, the first test compound. The remainder of steps in the assay are as set forth above.

In vitro assays such as those described above may be used advantageously to screen rapidly large numbers of compounds for effects on complex formation by ODAR and OPG binding protein. The assays may be automated to screen compounds generated in phage display, synthetic peptide and chemical synthesis libraries.

Compounds which increase or decrease complex formation of OPG binding protein and ODAR may also be screened in cell culture using ODAR-bearing cells and cell lines. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. ODAR containing cells such as osteoclasts may be enriched from other cell types by affinity chromatography using publicly available procedures. Attachment of OPG binding protein to ODAR-bearing cells is evaluated in the presence or absence of test compounds and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody to OPG binding protein. Alternatively, a mouse or human osteoclast

culture may be established as described in Example 8
and test compounds may be evaluated for their ability
to block osteoclast maturation stimulated by addition
of CSF-1 and OPG binding protein. Cell culture assays
5   may be used advantageously to further evaluate
compounds that score positive in protein binding assays
described above.

        Compounds which increase or decrease the
interaction of OPG binding protein with ODAR may also
10   be evaluated for _in vivo_ activity by administration of
the compounds to mice followed by measurements of bone
density using bone scanning densitometry or
radiography. Procedures for measuring bone density are
described in PCT publication WO97/23614 and in Example
15   13.

        The invention provides for compounds which
decrease or block the interaction of OPG binding
protein and ODAR and are antagonists of osteoclast
20   formation. Such compounds generally fall into two
groups. One group includes those compounds which are
derived from OPG binding protein or which interact with
OPG binding protein. These have been described above.
A second group includes those compounds which are
25   derived from ODAR or which interact with ODAR.
Examples of compounds which are antagonists of ODAR
include nucleic acids, proteins, peptides,
carbohydrates, lipids or small molecular weight organic
compounds.
30   Antagonists of ODAR may be compounds which
bind at or near one or more binding sites for OPG bp in
the ODAR extracellular domain and decrease or
completely block complex formation. Those regions on
ODAR that are involved in complex formation with OPG
35   binding protein may be identified by analogy with the

structure of the homologous TNFβ/TNF-R55 complex which has been described in Banner et al. (Cell $\underline{73}$, 431-445 (1993)). For example, the structure of the TNFβ/TNF-R55 complex may be used to identify regions of OPG
5   binding protein and ODAR that are involved in complex formation. Compounds may then be designed which preferentially bind to the regions involved in complex formation and act as antagonists. In one approach set forth in Example 11, peptide antigens were designed for
10   use in raising antibodies to OPG binding protein that act as antagonists. These antibodies are expected to bind to OPG binding protein and block complex formation with ODAR. In a similar approach, peptide antigens based upon ODAR structure may be used to raise anti-
15   ODAR antibodies that act as antagonists.

      Anatoginists of ODAR may also bind to ODAR at locations distinct from the binding site(s) for OPG bp and induce conformational changes in ODAR polypeptide that result in decreased or nonproductive complex
20   formation with OPG binding proteins.

      In one embodiment, an antagonist is a soluble form of ODAR lacking a functional transmembrane domain. Soluble forms of ODAR may have a deletion of one or
25   more amino acids in the transmembrane domain (amino acids 214-234 as shown in Figure 10). Soluble ODAR polypeptides may have part or all of the extracellular domain and are capable of binding OPG binding protein. Optionally, soluble ODAR may be part of a chimeric
30   protein, wherein part or all of the extracellular domain of ODAR is fused to a heterologous amino acid sequence. In one embodiment, the heterologous amino acid sequence is an Fc region from human IgG.

      Modulators (agonists and antagonists) of ODAR
35   may be used to prevent or treat osteopenia, including

osteoporosis, osteomyelitis, hypercalcemia of malignancy, osteopenia brought on by surgery or steroid administration, Paget's disease, osteonecrosis, bone loss due to rheumatoid arthritis, periodontal bone
5   loss, immobilization, prosthetic loosing and osteolytic metastasis. Agonists and antagonists of ODAR used for the treatment of osteopenias may be administered alone or in combination with a therapeutically effective amount of a bone growth promoting agent including bone
10 morphogenic factors designated BMP-1 to BMP-12, transforming growth factor-β and TGF-β family members, fibroblast growth factors FGF-1 to FGF-10, interleukin-1 inhibitors, TNFα inhibitors, parathyroid hormone, E series prostaglandins, bisphosphonates,
15 estrogens, SERMs and bone-enhancing minerals such as fluoride and calcium. Antagonists of ODAR are particularly useful in the treatment of osteopenia.

The following examples are offered to more
20 fully illustrate the invention, but are not construed as limiting the scope thereof.

Example 1
Identification of a cell line source for
25   an OPG binding protein

Osteoprotegerin (OPG) negatively regulates osteoclastogenesis in vitro and in vivo. Since OPG is a TNFR-related protein, it is likely to interact with a
30 TNF-related family member while mediating its effects. With one exception, all known members of the TNF superfamily are type II transmembrane proteins expressed on the cell surface. To identify a source of an OPG binding protein, recombinant OPG-Fc fusion
35 proteins were used as immunoprobes to screen for OPG

binding proteins located on the surface of various cell lines and primary hematopoietic cells.

Cell lines that grew as adherent cultures in vitro were treated using the following methods: Cells were plated into 24 well tissue culture plates (Falcon), then allowed to grow to approximately 80% confluency. The growth media was then removed, and the adherent cultures were washed with phosphate buffered saline (PBS) (Gibco) containing 1% fetal calf serum (FCS). Recombinant mouse OPG [22-194]-Fc and human OPG [22-201]-Fc fusion proteins (see U.S. Serial No. 08/706,945 filed September 3, 1996) were individually diluted to 5 ug/ml in PBS containing 1% FCS, then added to the cultures and allowed to incubate for 45 min at 0°C. The OPG-Fc fusion protein solution was discarded, and the cells were washed in PBS-FCS solution as described above. The cultures were then exposed to phycoeyrthrin-conguated goat F(ab') anti-human IgG secondary antibody (Southern Biotechnology Associates Cat. # 2043-09) diluted into PBS-FCS. After a 30-45 min incubation at 0°C, the solution was discarded, and the cultures were washed as described above. The cells were then analyzed by immunofluorescent microscopy to detect cell lines which express a cell surface OPG binding protein.

Suspension cell cultures were analyzed in a similar manner with the following modifications: The diluent and wash buffer consisted of calcium- and magnesium-free phosphate buffered saline containing 1% FCS. Cells were harvested from exponentially replicating cultures in growth media, pelleted by centrifugation, then resuspended at 1×10^7 cells/ml in a 96 well microtiter tissue culture plate (Falcon). Cells were sequentially exposed to recombinant OPG-Fc fusion proteins, then secondary antibody as described

above, and the cells were washed by centrifugation between each step. The cells were then analyzed by fluorescence activated cell sorting (FACS) using a Becton Dickinson FACscan.

5   Using this approach, the murine myelomonocytic cell line 32D (ATCC accession no. CRL-11346) was found to express a surface molecule which could be detected with both the mouse OPG[22-194]-Fc and the human OPG[22-201]-Fc fusion proteins.
10   Secondary antibody alone did not bind to the surface of 32D cells nor did purified human IgG1 Fc, indicating that binding of the OPG-Fc fusion proteins was due to the OPG moiety. This binding could be competed in a dose dependent manner by the addition of recombinant
15   murine or human OPG[22-401] protein. Thus the OPG region required for its biological activity is capable of specifically binding to a 32D-derived surface molecule.

20   Example 2
            Expression cloning of a murine OPG binding protein

            A cDNA library was prepared from 32D mRNA, and ligated into the mammalian expression vector
25   pcDNA3.1(+) (Invitrogen, San Diego, CA). Exponentially growing 32D cells maintained in the presence of recombinant interleukin-3 were harvested, and total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi.
30   Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (Dynal Corp) using the manufacturer's recommended procedures. A directional,
35   oligo-dT primed cDNA library was prepared using the

Superscript Plasmid System (Gibco BRL, Gaithersburg, Md) using the manufacturer's recommended procedures. The resulting cDNA was digested to completion with Sal I and Not I restriction endonuclease, then fractionated

5 by size exclusion gel chromatography. The highest molecular weight fractions were selected, and then ligated into the polyliker region of the plasmid vector pcDNA3.1(+) (Invitrogen, San Diego, CA). This vector contains the CMV promoter upstream of multiple cloning

10 site, and directs high level expression in eukaryotic cells. The library was then electroporated into competent E. coli (ElectroMAX DH10B, Gibco, NY), and titered on LB agar containing 100 ug/ml ampicillin. The library was then arrayed into segregated pools

15 containing approximately 1000 clones/pool, and 1.0 ml cultures of each pool were grown for 16-20 hr at 37°C. Plasmid DNA from each culture was prepared using the Qiagen Qiawell 96 Ultra Plasmid Kit (catalog #16191) following manufacturer's recommended procedures.

20   Arrayed pools of 32D cDNA expression library were individually lipofected into COS-7 cultures, then assayed for the acquisition of a cell surface OPG binding protein. To do this, COS-7 cells were plated at a density of 1×10^6 per ml in six-well tissue

25 culture plates (Costar), then cultured overnight in DMEM (Gibco) containing 10% FCS. Approximately 2 µg of plasmid DNA from each pool was diluted into 0.5 ml of serum-free DMEM, then sterilized by centrifugation through a 0.2 µm Spin-X column (Costar).

30 Simultaneously, 10 µl of Lipofectamine (Life Technologies Cat # 18324-012) was added to a separate tube containing 0.5ml of serum-free DMEM. The DNA and Lipofectamine solutions were mixed, and allowed to incubate at RT for 30 min. The COS-7 cell cultures

35 were then washed with serum-free DMEM, and the DNA-

lipofectamine complexes were exposed to the cultures for 2-5 hr at 37°C. After this period, the media was removed, and replaced with DMEM containing 10%FCS. The cells were then cultured for 48 hr at 37°C.

5   To detect cultures that express an OPG binding protein, the growth media was removed, and the cells were washed with PBS-FCS solution. A 1.0 ml volume of PBS-FCS containing 5 µg/ml of human OPG[22-201]-Fc fusion protein was added to each well and

10   incubated at RT for 1 hr. The cells were washed three times with PBS-FCS solution, and then fixed in PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde in PBS at RT for 5 min. The cultures were washed once with PBS-FCS, then incubated for 1 hr at 65°C while

15   immersed in PBS-FCS solution. The cultures were allowed to cool, and the PBS-FCS solution was aspirated. The cultures were then incubated with an alkaline-phosphatase conjugated goat anti-human IgG (Fc specific) antibody (SIGMA Product # A-9544) at Rt for

20   30 min, then washed three-times with 20 mM Tris-Cl (pH 7.6), and 137 mM NaCl. Immune complexes that formed during these steps were detected by assaying for alkaline phosphatase activity using the Fast Red TR/AS-MX Substrate Kit (Pierce, Cat. # 34034) following the

25   manufacturer's recommended procedures.

       Using this approach, a total of approximately 300,000 independent 32D cDNA clones were screened, represented by 300 transfected pools of 1000 clones each. A single well was identified that contained

30   cells which acquired the ability to be specifically decorated by the OPG-Fc fusion protein. This pool was subdivided by sequential rounds of sib selection, yielding a single plasmid clone 32D-F3 (Figure 1). 32D-F3 plasmid DNA was then transfected into COS-7

35   cells, which were immunostained with either FITC-

conjugated goat anti-human IgG secondary antibody
alone, human OPG[22-201]-Fc fusion protein plus
secondary, or with ATAR-Fc fusion protein (ATAR also
known as HVEM; Montgomery et al. Cell 87, 427-436

5   (1996)) (Figure 2). The secondary antibody alone did
not bind to COS-7/32D-F3 cells, nor did the ATAR-Fc
fusion protein. Only the OPG Fc fusion protein bound
to the COS-7/32D-F3 cells, indicating that 32D-F3
encoded an OPG binding protein displayed on the surface

10   of expressing cells.

Example 3

OPG Binding Protein Sequence

15   The 32D-F3 clone isolated above contained an
approximately 2.3 kb cDNA insert (Figure 1), which was
sequenced in both directions on an Applied Biosystems
373A automated DNA sequencer using primer-driven Taq
dye-terminator reactions (Applied Biosystems) following

20   the manufacturer's recommended procedures. The
resulting nucleotide sequence obtained was compared to
the DNA sequence database using the FASTA program (GCG,
University of Wisconsin), and analyzed for the presence
of long open reading frames (LORF's) using the "Six-way

25   open reading frame" application (Frames) (GCG,
University of Wisconsin). A LORF of 316 amino acid
(aa) residues beginning at methionine was detected in
the appropriate orientation, and was preceded by a 5'
untranslated region of about 150 bp. The 5'

30   untranslated region contained an in-frame stop codon
upstream of the predicted start codon. This indicates
that the structure of the 32D-F3 plasmid is consistent
with its ability to utilize the CMV promoter region to
direct expression of a 316 aa gene product in mammalian

35   cells.

The predicted OPG binding protein sequence
was then compared to the existing database of known
protein sequences using a modified version of the FASTA
program (Pearson, Meth. Enzymol. 183, 63-98 (1990)).
5   The amino acid sequence was also analyzed for the
presence of specific motifs conserved in all known
members of the tumor necrosis factor (TNF) superfamily
using the sequence profile method of (Gribskov et al.
Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as
10   modified by Lüethy et al. Protein Sci. 3, 139-146
(1994)). There appeared to be significant homology
throughout the OPG binding protein to several members
of the TNF superfamily. The mouse OPG binding protein
appear to be most closely related to the mouse and
15   human homologs of both TRAIL and CD40 ligand. Further
analysis of the OPG binding protein sequence indicated
a strong match to the TNF superfamily, with a highly
significant Z score of 19.46.

The OPG binding protein amino acid sequence
20   contains a probable hydrophobic transmembrane domain
that begins at a M49 and extends to L69. Based on this
configuration relative to the methionine start codon,
the OPG binding protein is predicted to be a type II
transmembrane protein, with a short N-terminal
25   intracellular domain, and a longer C-terminal
extracellular domain (Figure 4). This would be similar
to all known TNF family members, with the exception of
lymphotoxin alpha (Nagata and Golstein, Science 267,
1449-1456 (1995)).
30

<div align="center">

Example 4

Expression of human OPG binding protein mRNA

</div>

Multiple human tissue northern blots
35   (Clontech, Palo Alto, CA) were probed with a ^{32}P-dCTP

labeled 32D-F3 restriction fragment to detect the size
of the human transcript and to determine patterns of
expression. Northern blots were prehybridized in 5X
SSPE, 50% formamide, 5X Denhardt's solution, 0.5% SDS,
5   and 100 µg/ml denatured salmon sperm DNA for 2-4 hr at
42°C. The blots were then hybridized in 5X SSPE, 50%
formamide, 2X Denhardt's solution, 0.1% SDS, 100 µg/ml
denatured salmon sperm DNA, and 5 ng/ml labeled probe
for 18-24 hr at 42°C. The blots were then washed in 2X
10   SSC for 10 min at RT, 1X SSC for 10 min at 50°C, then
in 0.5X SSC for 10-15 min.

Using a probe derived from the mouse cDNA and
hybridization under stringent conditions, a predominant
mRNA species with a relative molecular mass of about
15   2.5 kb was detected in lymph nodes (Figure 3). A faint
signal was also detected at the same relative molecular
mass in fetal liver mRNA. No OPG binding protein
transcripts were detected in the other tissues
examined. The data suggest that expression of OPG
20   binding protein mRNA was extremely restricted in human
tissues. The data also indicate that the cDNA clone
isolated is very close to the size of the native
transcript, suggesting 32D-F3 is a full length clone.

25   Example 5
        Molecular cloning of the human OPG binding protein

The human homolog of the OPG binding protein
is expressed as an approximately 2.5 kb mRNA in human
30   peripheral lymph nodes and is detected by hybridization
with a mouse cDNA probe under stringent hybridization
conditions. DNA encoding human OPG binding protein is
obtained by screening a human lymph node cDNA library
by either recombinant bacteriphage plaque, or
35   transformed bacterial colony, hybridization methods

(Sambrook et al. <u>Molecular Cloning: A Laboratory Manual</u>
Cold Spring Harbor Press, New York (1989)). To this
the phage or plasmid cDNA library are screened using
radioactively-labeled probes derived from the murine
5   OPG binding protein clone 32D-F3. The probes are used
to screen nitrocellulose filter lifted from a plated
library. These filters are prehybridized and then
hybridized using conditions specified in Example 4,
ultimately giving rise to purified clones of the human
10 OPG binding protein cDNA. Inserts obtained from any
human OPG binding protein clones would be sequenced and
analyzed as described in Example 3.

A human lymph node poly A+ RNA (Clontech,
Inc., Palo Alto, CA) was analyzed for the presence of
15 OPG-bp transcripts as previously in U.S. Serial No.
08/577,788, filed December 22, 1995. A northern blot
of this RNA sample probed under stringent conditions
with a 32P-labeled mouse OPG-bp probe indicated the
presence of human OPG-bp transcripts. An oligo dT-
20 primed cDNA library was then synthesized from the lymph
node mRNA using the SuperScript kit (GIBCO life
Technologies, Gaithersberg, MD) as described in example
2. The resulting cDNA was size selected, and the high
molecular fraction ligated to plasmid vector pcDNA 3.1
25 (+) (Invitrogen, San Diego, CA). Electrocompetent <u>E.</u>
<u>coli</u> DH10 (GIBCO life Technologies, Gaithersberg, MD)
were transformed, and 1 X 10^6 ampicillin resistant
transformants were screened by colony hybridization
using a 32P-labeled mouse OPG binding protein probe.
30   A plasmid clone of putative human OPG binding
protein cDNA was isolated, phuOPGbp-1.1, and contained
a 2.3 kp insert. The resulting nucleotide sequence of
the phuOPGbp-1.1 insert was approximately 80-85%
homologous to the mouse OPG binding protein cDNA
35 sequence. Translation of the insert DNA sequence

indicated the presence of a long open reading frame predicted to encode a 317 aa polypeptide (Figure 4). Comparison of the mouse and human OPG-bp polypeptides shows that they are ~87% identical, indicating that

5   this protein is highly conserved during evolution.

The human OPG binding protein DNA and protein sequences were not present in Genbank, and there were no homologus EST sequences. As with the murine homolog, the human OPG binding protein shows strong

10   sequence similarity to all members of the TNFα superfamily of cytokines.

Example 6

Cloning and Bacterial Expression of OPG binding protein

15

PCR amplification employing the primer pairs and templates described below are used to generate various forms of murine OPG binding proteins. One primer of each pair introduces a TAA stop codon and a

20   unique XhoI or SacII site following the carboxy terminus of the gene. The other primer of each pair introduces a unique NdeI site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling is performed using

25   standard recombinant DNA methodology. The PCR products are purified, restriction digested, and inserted into the unique NdeI and XhoI or SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic E. coli 393 or 2596. Other commonly

30   used E. coli expression vectors and host cells are also suitable for expression. After transformation, the clones are selected, plasmid DNA is isolated and the sequence of the OPG binding protein insert is confirmed.

35

pAMG21-Murine OPG binding protein [75-316]

This construct was engineered to be 242 amino acids in length and have the following N-terminal and C-terminal residues, NH$_2$-Met(75)-Asp-Pro-Asn-Arg-------Gln-Asp-Ile-Asp(316)-COOH. The template to be used for PCR was pcDNA/32D-F3 and oligonucleotides #1581-72 and #1581-76 were the primer pair to be used for PCR and cloning this gene construct.

1581-72:
  5'-GTTCTCCTCATATGGATCCAAACCGTATTTCTGAAGACAGCACTCACTGCTT-3'
      (SEQ ID NO: 5)
1581-76:
  5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'
      (SEQ ID NO: 6)

pAMG21-Murine OPG binding protein [95-316]

This construct was engineered to be 223 amino acids in length and have the following N-terminal and C-terminal residues, NH$_2$-Met-His(95)-Glu-Asn-Ala-Gly-------Gln-Asp-Ile-Asp(316)-COOH. The template used for PCR was pcDNA/32D-F3 and oligonucleotides #1591-90 and #1591-95 were the primer pair used for PCR and cloning this gene construct.

1591-90:
  5'-ATTTGATTCTAGAAGGAGGAATAACATATGCATGAAAACGCAGGTCTGCAG-3'
      (SEQ ID NO: 7)
1591-95:
  5'-TATCCGCGGATCCTCGAGTTAGTCTATGTCCTGAACTTTGAA-3'
      (SEQ ID NO: 8)

pAMG21-Murine OPG binding protein [107-316]

This construct was engineered to be 211 amino acids in length and have the following N-terminal and C-terminal residues, NH$_2$-Met-Ser(107)-Glu-Asp-Thr-Leu--

-----Gln-Asp-Ile-Asp(316)-COOH. The template used for PCR was pcDNA/32D-F3 and oligonucleotides #1591-93 and #1591-95 were the primer pair used for PCR and cloning this gene construct.

5

1591-93:
    5'-ATTTGATTCTAGAAGGAGGAATAACATATGTCTGAAGACACTCTGCCGGACTCC-3'
      (SEQ ID NO: 9)

5   1591-95:
    5'-TATCCGCGGATCCTCGAGTTAGTCTATGTCCTGAACTTTGAA-3'
      (SEQ ID NO: 10)

10   <u>pAMG21-Murine OPG binding protein [118-316]</u>

This construct was engineered to be 199 amino acids in length and have the following N-terminal and C-terminal residues, NH_2-Met(118)-Lys-Gln-Ala-Phe-Gln-------Gln-Asp-Ile-Asp(316)-COOH. The template used for

15   PCR was pcDNA/32D-F3 and oligonucleotides #1591-94 and #1591-95 were the primer pair used for PCR and cloning this gene construct.

1591-94:

20   5'-ATTTGATTCTAGAAGGAGGAATAACATATGAAACAAGCTTTTCAGGGG-3'
      (SEQ ID NO: 11)
    1591-95:
    5'-TATCCGCGGATCCTCGAGTTAGTCTATGTCCTGAACTTTGAA-3'
      (SEQ ID NO: 12)

25

<u>pAMG21-Murine OPG binding protein [128-316]</u>

This construct was engineered to be 190 amino acids in length and have the following N-terminal and C-terminal residues, NH_2-Met-Lys(128)-Glu-Leu-Gln-His-------Gln-Asp-Ile-Asp(316)-COOH. The template used for

30   PCR was pcDNA/32D-F3 and oligonucleotides #1591-91 and #1591-95 were the primer pair used for PCR and cloning this gene construct.

35   1591-91:
    5'-ATTTGATTCTAGAAGGAGGAATAACATATGAAAGAACTGCAGCACATTGTG-3'
      (SEQ ID NO: 13)

1591-95:
    5'-TATCCGCGGATCCTCGAGTTAGTCTATGTCCTGAACTTTGAA-3'
        (SEQ ID NO: 14)

5   pAMG21-Murine OPG binding protein [137-316]

           This construct was engineered to be 181 amino
    acids in length and have the following N-terminal and
    C-terminal residues, NH_2-Met-Gln(137)-Arg-Phe-Ser-Gly--
    -----Gln-Asp-Ile-Asp(316)-COOH. The template used for
10 PCR was pcDNA/32D-F3 and oligonucleotides #1591-92 and
    #1591-95 were the primer pair used for PCR and cloning
    this gene construct.

    1591-92:
15   5'-ATTTGATTCTAGAAGGAGGAATAACATATGCAGCGTTTCTCTGGTGCTCCA-3'
            (SEQ ID NO: 15)
    1591-95:
        5'-TATCCGCGGATCCTCGAGTTAGTCTATGTCCTGAACTTTGAA-3'
            (SEQ ID NO: 16)
20

    pAMG21-Murine OPG binding protein [146-316]

           This construct is engineered to be 171 amino
    acids in length and have the following N-terminal and
    C-terminal residues, NH_2-Met(146)-Glu-Gly-Ser-Trp------
25 --Gln-Asp-Ile-Asp(316)- COOH. The template to be used
    for PCR is pAMG21-murine OPG binding protein [75-316]
    described above and oligonucleotides #1600-98 and
    #1581-76 will be the primer pair to be used for PCR and
    cloning this gene construct.
30
    1600-98:
        5'- GTTCTCCTCATATGGAAGGTTCTTGGTTGGATGTGGCCCA-3'
            (SEQ ID NO: 17)
    1581-76:
35   5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'
            (SEQ ID NO: 18)

<u>pAMG21-Murine OPG binding protein [156-316]</u>

This construct is engineered to be 162 amino acids in length and have the following N-terminal and C-terminal residues, NH_2-Met-Arg(156)-Gly-Lys-Pro------

5 --Gln-Asp-Ile-Asp(316)- COOH. The template to be used for PCR is pAMG21-murine OPG binding protein [158-316] below and oligonucleotides #1619-86 and #1581-76 will be the primer pair to be used for PCR and cloning this gene construct.

10

1619-86:
  5'- GTTCTCCTCATATGCGTGGTAAACCTGAAGCTCAACCATTTGCA-3'
    (SEQ ID NO: 19)
1581-76:
15   5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'
    (SEQ ID NO: 20)

<u>pAMG21-Murine OPG binding protein [158-316]</u>

This construct was engineered to be 160 amino
20 acids in length and have the following N-terminal and C-terminal residues, NH_2-Met-Lys(158)-Pro-Glu-Ala------ --Gln-Asp-Ile-Asp(316)- COOH. The template to be used for PCR was pcDNA/32D-F3 and oligonucleotides #1581-73 and #1581-76 were the primer pair to be used for PCR
25 and cloning this gene construct.

1581-73:
  5'-GTTCTCCTCATATGAAACCTGAAGCTCAACCATTTGCACACCTCACCATCAAT-3'
    (SEQ ID NO: 21)
30 1581-76:
  5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'
    (SEQ ID NO: 22)

<u>pAMG21-Murine OPG binding protein [166-316]</u>

35 This construct is engineered to be 152 amino acids in length and have the following N-terminal and

C-terminal residues, NH$_2$-Met-His(166)-Leu-Thr-Ile------
--Gln-Asp-Ile-Asp(316)- COOH. The template to be used
for PCR is pcDNA/32D-F3 and oligonucleotides #1581-75
and #1581-76 will be the primer pair to be used for PCR
and cloning this gene construct.

1581-75:
5'-GTTCTCCTCATATGCATTTAACTATTAACGCTGCATCTATCCCAT
    CGGGTTCCCATAAAGTCACT-3'   (SEQ ID NO: 23)
1581-76:
5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'   (SEQ ID NO: 24)

pAMG21-Murine OPG binding protein [168-316]

        This construct is engineered to be 150 amino
acids in length and have the following N-terminal and
C-terminal residues, NH$_2$-Met-Thr(168)-Ile-Asn-Ala------
--Gln-Asp-Ile-Asp(316)- COOH. The template to be used
for PCR is pcDNA/32D-F3 and oligonucleotides #1581-74
and #1581-76 will be the primer pair to be used for PCR
and cloning.

1581-74:
5'-GTTCTCCTCATATGACTATTAACGCTGCATCTATCCCATCGGGTTCCCATAAAGTCACT-3'
    (SEQ ID NO: 25)
1581-76:
5'-TACGCACTCCGCGGTTAGTCTATGTCCTGAACTTTGA-3'   (SEQ ID NO: 26)

It is understood that the above constructs are examples
and one skilled in the art may readily obtain other
forms of OPG binding protein using the general
methodology presented her.
        Recombinant bacterial constructs pAMG21-
murine OPG binding protein [75-316], [95-316], [107-
316], [118-316], [128-316], [137-316], and [158-316]
have been cloned, DNA sequence confirmed, and levels of
recombinant gene product expression following induction

has been examined. All constructs produced levels of recombinant gene product which was readily visible following SDS polyacrylamide gel electrophoresis and coomassie staining of crude lysates. Growth of

5   transformed <u>E. coli</u> 393 or 2596, induction of OPG binding protein expression and isolation of inclusion bodies containing OPG binding protein is done according to procedures described in PCT WO97/23614. Purification of OPG binding proteins from inclusion

10   bodies requires solubilization and renaturing of OPG binding protein using procedures available to one skilled in the art. Recombinant murine OPG binding protein [158-316] was found to be produced mostly insolubly, but about 40% was found in the soluble

15   fraction. Recombinant protein was purified from the soluble fraction as described below and its bioactivity examined.

<div align="center">

Example 7

20   Purification of recombinant murine
OPG binding protein [158-316]

</div>

    Frozen bacterial cells harboring expressed murine OPG binding protein (158-316) were thawed and

25   resuspended in 20mM tris-HCl pH 7.0, 10mM EDTA. The cell suspension (20%w/v) was then homogenized by three passes through a microfluidizer. The lysed cell suspension was centrifuged in a JA14 rotor at 10,000 rpm for 45 minutes. SDS-PAGE analysis showed a band of

30   approximately 18kd molecular weight present in both inclusion bodies and the supernatant. The soluble fraction was then applied to a Pharmacia SP Sepharose 4FF column equilibrated with 10mM MES pH 6.0. The OPG binding protein was eluted with a 20 column volume

35   gradient of 0-0.4M NaCl in MES pH 6.0. Fractions

containing OPG binding protein were then applied to an
ABX Bakerbond column equilibrated with 20mM MES pH 6.0.
OPG binding protein was eluted with a 15CV gradient of
0-0.5M NaCl in MES pH 6.0. The final product was over
5 95% homogeneous by SDS-PAGE. N-terminal sequencing gave
the following sequence: Met-Lys-Pro-Glu-Ala-Gln-Pro-
Phe-Ala-His which was identified to that predicted for
a polypeptide starting at residue 158 (with an
initiator methionine). The relative molecular weight
10 of the protein during SDS-PAGE does not change upon
reduction.

Example 8
In vitro bioactivity of recombinant soluble
15   OPG binding protein

Recombinant OPG protein has previously been
shown to block vitamin D3-dependent osteoclast
formation from bone marrow and spleen precursors in an
20 osteoclast forming assay as described in U.S. Serial
No. 08/577,788. Since OPG binding protein binds to
OPG, and is a novel member of the TNF family of
ligands, it is a potential target of OPG bioactivity.
Recombinant soluble OPG binding protein (158-316),
25 representing the minimal core TNFα-like domain, was
tested for its ability to modulate osteoclast
differentiation from osteoclast precursors. Bone
marrow cells were isolated from adult mouse femurs, and
treated with M-CSF. The non-adherent fraction was co-
30 cultured with ST2 cells in the presence and absence of
both vitamin D3 and dexamethasone. As previously
shown, osteoclasts develop only from co-cultures
containing stromal cells (ST2), vitamin D3 and
dexamethasone. Recombinant soluble OPG binding protein
35 was added at varying concentrations ranging from 0.16

to 500 ng/ml and osteoclast maturation was determined
by TRAP solution assay and by visual observation. OPG
binding protein strongly stimulated osteoclast
differentiation and maturation in a dose dependent
manner, with half-maximal effects in the 1-2 ng/ml
range, suggesting that it acts as an potent inducer of
osteoclastogenesis in vitro (Figure 5). The effect of
OPG binding protein is blocked by recombinant OPG
(Figure 6).

To test whether OPG binding protein could
replace the stroma and added steroids, cultures were
established using M-CSF at varying concentrations to
promote the growth of osteoclast precursors and various
amounts of OPG binding protein were also added. As
shown in Figure 6, OPG binding protein dose dependently
stimulated TRAP activity, and the magnitude of the
stimulation was dependent on the level of added M-CSF
suggesting that these two factors together are pivotal
for osteoclast development. To confirm the biological
relevance of this last observation, cultures were
established on bovine cortical bone slices and the
effects of M-CSF and OPG binding protein either alone
or together were tested. As shown in Figure 7, OPG
binding protein in the presence of M-CSF stimulated the
formation of large TRAP positive osteoclasts that
eroded the bone surface resulting in pits. Thus, OPG
binding protein acts as an osteoclastogenesis
stimulating (differentiation) factor. This suggests
that OPG blocks osteoclast development by sequestering
OPG binding protein.

Example 9
In vivo activity of recombinant
soluble OPG Binding Protein

Based on in vitro studies, recombinant murine OPG binding protein [158-316] produced in <u>E.coli</u> is a potent inducer of osteoclast development from myeloid precursors. To determine its effects in vivo, male BDF1 mice aged 4-5 weeks (Charles River Laboratories) received subcutaneous injections of OPG binding protein [158-316] twice a day for three days and on the morning of the fourth day (days 0, 1, 2, and 3). Five groups of mice (n=4) received carrier alone, or 1, 5, 25 or 100μg/ of OPG binding protein [158-316] per day. An additional 5 groups of mice (n=4) received the above doses of carrier or of OPG binding protein [158-316] and in addition received human Fc-OPG [22-194] at 1mg/Kg/day (approximately 20 μg/day) by single daily subcutaneous injection. Whole blood ionized calcium was determined prior to treatment on day 0 and 3-4 hours after the first daily injection of OPG binding protein [158-316] on days 1, 2, and 3. Four hours after the last injection on day 3 the mice were sacrificed and radiographs were taken.

Recombinant of OPG binding protein [158-316] produced a significant increase in blood ionized calcium after two days of treatment at dose of 5 μg/day and higher (Figure 8). The severity of the hypercalcemia indicates a potent induction of osteoclast activity resulting from increased bone resorption. Concurrent OPG administration limited hypercalcemia at doses of OPG binding protein [158-316] of 5 and 25 μg/day, but not at 100 μg/day. These same animal were analyzed by radiography to determine if there were any effects on bone mineral density visible by X-ray (Figure 9). Recombinant of OPG binding protein [158-316] injected for 3 days decreased bone density in the proximal tibia of mice in a dose-

dependent manner. The reduction in bone density was particularly evident in mice receiving 100 µg/d confirming that the profound hypercalcemia in these animals was produced from increased bone resorption and the resulting release of calcium from the skeleton. These data clearly indicate that of OPG binding protein [158-316] acts in vivo to promote bone resorption, leading to systemic hypercalcemia, and recombinant OPG abbrogates these effects.

Example 10
Cloning and Expression of soluble OPG Binding Protein in mammalian cells

The full length clone of murine and human OPG binding protein can be expressed in mammalian cells as previously described in Example 2. Alternatively, the cDNA clones can be modified to encode secreted forms of the protein when expressed in mammalian cells. To do this, the natural 5'end of the cDNA encoding the initiation codon, and extending approximately through the first 69 amino acid of the protein, including the transmembrane spanning region, could be replaced with a signal peptide leader sequence. For example, DNA sequences encoding the initiation codon and signal peptide of a known gene can be spliced to the OPG binding protein cDNA sequence beginning anywhere after the region encoding amino acid residue 68. The resulting recombinant clones are predicted to produce secreted forms of OPB binding protein in mammalian cells, and should undergo post translational modifications which normally occur in the C-terminal extracellular domain of OPG binding protein, such as glycoslyation. Using this strategy, a secreted form of OPG binding protein was constructed which has at its 5'

end the murine OPG signal peptide, and at its 3' end
the human IgG1 Fc domain. The plasmid vector
pCEP4/muOPG[22-401]-Fc as described in U.S. Serial No.
08/577,788, filed December 22, 1995, was digested with
5   NotI to cleave between the 3' end of OPG and the Fc
gene. The linearized DNA was then partially digested
with XmnI to cleave only between residues 23 and 24 of
OPG leaving a blunt end. The restriction digests were
then dephosphorylated with CIP and the vector portion
10 of this digest (including residues 1-23 of OPG and Fc)
was gel purified.

          The murine OPG binding protein cDNA region
encoding amino acid residues 69-316 were PCR amplified
using Pfu Polymerase (Stratagene, San Diego, CA) from
15 the plasmid template using primers the following
oligonucleotides:
1602-61: CCT CTA GGC CTG TAC TTT CGA GCG CAG ATG (SEQ
ID NO: 27)

20 1602-59: CCT CTG CGG CCG CGT CTA TGT CCT GAA CTT TG
(SEQ ID NO: 28)
          The 1602-61 oligonucleotide amplifies the 5'
end of the gene and contains an artificial an StuI
site. The 1602-59 primer amplifies the 3' end of the
25 gene and contains an artificial NotI site. The
resulting PCR product obtained was digested with NotI
and StuI, then gel purified. The purified PCR product
was ligated with vector, then used to transform
electrocompetent E. coli DH10B cells. The resulting
30 clone was sequenced to confirm the integrity of the
amplified sequence and restriction site junctions.
This plasmid was then used to transfect human 293
fibroblasts, and the OPG binding protein-Fc fusion
protein was collected form culture media as previously

described in U.S. Serial No. 08/577,788, filed December
22, 1995.

Using a similar strategy, an expression
vector was designed that is capable of expressing a N-
5   terminal truncation of fused to the human IgG1 Fc
domain. This construct consists of the murine OPG
signal peptide (aa residue 1-21), fused in frame to
murine OPG binding protein residues 158-316, followed
by an inframe fusion to human IgG1 Fc domain. To do
10   this, the plasmid vector pCEP4/ murine OPG [22-401]
(U.S. Serial No. 08/577,788, filed December 22, 1995),
was digested with HindIII and NotI to remove the entire
OPG reading frame. Murine OPG binding protein,
residues 158-316 were PCR amplified using from the
15   plasmid template pCDNA/32D-F3 using the following
primers:
1616-44: CCT CTC TCG AGT GGA CAA CCC AGA AGC CTG AGG
CCC AGC CAT TTG C (SEQ ID NO: 29)
1602-59: CCT CTG CGG CCG CGT CTA TGT CCT GAA CTT TG
20   (SEQ ID NO: 30)
1616-44 amplifies OPG binding protein
starting at residue 158 as well as containing residues
16-21 of the muOPG signal peptide with an artificial
XhoI site. 1602-59 amplifies the 3' end of the gene
25   and adds an in-frame NotI site. The PCR product was
digested with NotI and XhoI and then gel purified.
The following complimentary primers were
annealed to eachother to form an adapter encoding the
murine OPG signal peptide and Kozak sequence
30   surrounding the translation initiation site:

1616-41: AGC TTC CAC CAT GAA CAA GTG GCT GTG CTG CGC
ACT CCT GGT GCT CCT GGA CAT CA (SEQ ID NO: 31)

1616-42: TCG ATG ATG TCC AGG AGC ACC AGG AGT GCG CAG CAC AGC CAC TTG TTC ATG GTG GA (SEQ ID NO: 32)

5   These primers were annealed, generating 5′ overhangs compatible with HindIII on the 5′ end and XhoI on the 3′ end. The digested vector obtained above, the annealed oligos, and the digested PCR fragment were ligated together and electroporated into DH10B cells. The resulting clone was sequenced to

10   confirm authentic reconstruction of the junction between the signal peptide, OPG binding protein fragment encoding residues 158-316, and the IgG1 Fc domain. The recombinant plasmid was purified, transfected into human 293 fibroblasts, and expressed

15   as a conditioned media product as described above.

     Full length murine and human cDNAs were cloned into the pCEP4 expression vector (Invitrogen, San Diego, CA) then transfected into cultures of human

20   293 fibroblasts as described in Example 1. The cell cultures were selected with hygromycin as described above and serum-free conditioned media was prepared. The conditioned media was exposed to a column of immobilized recombinant OPG, and shed forms of murine

25   and human recombinant OPG bp were affinity purified. N-terminal sequence analysis of the purified soluble OPG binding proteins indicates that the murine protein is preferentially cleaved before phenylalanine 139, and the human protein is preferentially cleaved before the

30   homologous residue, isoleucine 140. In addition the human protein is also preferentially cleaved before glycine 145. This suggests that naturally occurring soluble forms of human OPG binding protein have amino terminal residues at either isoleucine at position 140

35   or glycine at position 145.

Example 11
Peptides of the OPG binding protein and preparation of
polyclonal and monoclonal antibodies to the protein

Antibodies to specific regions of the OPG
binding protein may be obtained by immunization with
peptides from OPG binding protein. These peptides may
be used alone, or conjugated forms of the peptide may
be used for immunization.

The crystal structure of mature TNFα has been
described [E.Y. Jones, D.I. Stuart, and N.P.C. Walker
(1990) J. Cell Sci. Suppl. 13, 11-18] and the monomer
forms an antiparallel β-pleated sheet sandwich with a
jellyroll topology. Ten antiparallel β-strands are
observed in this crystal structure and form a beta
sandwich with one beta sheet consisting of strands
B'BIDG and the other of strands C'CHEF [E.Y. Jones et
al., ibid.] Two loops of mature TNFα have been
implicated from mutagenesis studies to make contacts
with receptor, these being the loops formed between
beta strand B & B' and the loop between beta strands E
& F [C.R. Goh, C-S. Loh, and A.G. Porter (1991) Protein
Engineering 4, 785-791]. The crystal structure of the
complex formed between TNFβ and the extracellular
domain of the 55kd TNF receptor (TNF-R55) has been
solved and the receptor-ligand contacts have been
described [D.W. Banner, A. D'Arcy, W. Janes, R. Gentz,
H-J. Schoenfeld, C. Broger, H. Loetscher, and W.
Lesslauer (1993) Cell 73, 431-445]. In agreement with
mutagenesis studies described above [C.R. Goh et al.,
ibid.] the corresponding loops BB' and EF of the ligand
TNFβ were found to make the majority of contacts with
the receptor in the resolved crystal structure of the

TNFb:TNF-R55 complex. The amino acid sequence of murine OPG binding protein was compared to the amino acid sequences of TNFα and TNFβ. The regions of murine OPG binding protein corresponding to the BB' and EF loops were predicted based on this comparison and peptides have been designed and are described below

A. <u>Antigen(s)</u>: Recombinant murine OPG binding protein [158-316] has been used as an antigen (ag) for immunization of animals as described below, and serum will be examined using approaches described below. Peptides to the putative BB' and EF loops of murine OPG binding protein have been synthesized and will be used for immunization; these peptides are:

BB' loop peptide: NH2--NAASIPSGSHKVTLSSWYHDRGWAKIS--COOH (SEQ ID NO: 33)
BB' loop-Cys peptide: NH2--NAASIPSGSHKVTLSSWYHDRGWAKISC--COOH (SEQ ID NO: 34)
EF loop peptide: NH2--VYVVKTSIKIPSSHNLM--COOH (SEQ ID NO: 35)
EF loop-Cys peptide: NH2--VYVVKTSIKIPSSHNLMC--COOH (SEQ ID NO: 36)

Peptides with a carboxy-terminal cysteine residue have been used for conjugation using approaches described in section B below, and have been used for immunization.

B. <u>Keyhole Limpet Hemocyanin or Bovine Serum Albumin Conjugation</u>: Selected peptides or protein fragments may be conjugated to keyhole limpet hemocyanin (KLH) in order to increase their immunogenicity in animals. Also, bovine serum albumin (BSA) conjugated peptides or protein fragments may be utilized in the EIA protocol. Imject Maleimide Activated KLH or BSA (Pierce Chemical Company, Rockford, IL) is reconstituted in dH$_2$O to a final concentration of 10 mg/ml. Peptide or protein

fragments are dissolved in phosphate buffer then mixed
with an equivalent mass (g/g) of KLH or BSA. The
conjugation is allowed to react for 2 hours at room
temperature (rt) with gentle stirring. The solution is
5   then passed over a desalting column or dialyzed against
PBS overnight. The peptide conjugate is stored at -
20°C until used in immunizations or in EIAs.

     C. Immunization: Balb/c mice, (Charles
Rivers Laboratories, Wilmington, MA) Lou rats, or New
10   Zealand White rabbits will be subcutaneously injected
(SQI) with ag (50 μg, 150 μg, and 100 μg respectively)
emulsified in Complete Freund's Adjuvant (CFA, 50%
vol/vol; Difco Laboratories, Detroit, MI). Rabbits are
then boosted two or three times at 2 week intervals
15   with antigen prepared in similar fashion in Incomplete
Freund's Adjuvant (ICFA; Difco Laboratories, Detroit,
MI). Mice and rats are boosted approximately every 4
weeks. Seven days following the second boost, test
bleeds are performed and serum antibody titers
20   determined. When a titer has developed in rabbits,
weekly production bleeds of 50 mls are taken for 6
consecutive weeks. Mice and rats are selected for
hybridoma production based on serum titer levels;
animals with half-maximal titers greater than 5000 are
25   used. Adjustments to this protocol may be applied by
one skilled in the art; for example, various types of
immunomodulators are now available and may be
incorporated into this protocol.

     D. Enzyme-linked Immunosorbent Assay (EIA):
30   EIAs will be performed to determine serum antibody (ab)
titres of individual animals, and later for the
screening of potential hybridomas. Flat bottom, high-
binding, 96-well microtitration EIA/RIA plates (Costar
Corporation, Cambridge, MA) will be coated with
35   purified recombinant protein or protein fragment

(antigen, ag) at 5 μg per ml in carbonate-bicarbonate buffer, pH 9.2 (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$). Protein fragments may be conjugated to bovine serum albumin (BSA) if necessary. Fifty μl of ag will be

5   added to each well. Plates will then be covered with acetate film (ICN Biomedicals, Inc., Costa Mesa, CA) and incubated at room temperature (rt) on a rocking platform for 2 hours or over-night at 4°C. Plates will be blocked for 30 minutes at rt with 250 μl per well 5%

10   BSA solution prepared by mixing 1 part BSA diluent/blocking solution concentrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) with 1 part deionized water (dH_2O). Blocking solution having been discarded, 50 μl of serum 2-fold dilutions (1:100

15   through 1: 12,800) or hybridoma tissue culture supernatants will be added to each well. Serum diluent is 1% BSA (10% BSA diluent/blocking solution concentrate diluted 1:10 in Dulbecco's Phosphate Buffered Saline, D-PBS; Gibco BRL, Grand Island, NY))

20   while hybridoma supernatants are tested undiluted. In the case of hybridoma screening, one well is maintained as a conjugate control, and a second well as a positive ab control. Plates are again incubated at rt, rocking for 1 hour, then washed 4 times using a 1x preparation

25   of wash solution 20x concentrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in dH_2O. Horseradish peroxidase conjugated secondary ab (Boeringer Mannheim Biochemicals, Indianapolis, IN) diluted in 1% BSA is then incubated in each well for 30

30   minutes. Plates are washed as before, blotted dry, and ABTS peroxidase single component substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) is added. Absorbance is read at 405 nm for each well using a Microplate EL310 reader (Bio-tek Instruments,

35   Inc., Winooski, VT). Half-maximal titre of serum

antibody is calculated by plotting the $\log_{10}$ of the serum dilution versus the optical density at 405, then extrapolating at the 50% point of the maximal optical density obtained by that serum. Hybridomas are

5   selected as positive if optical density scores greater than 5-fold above background. Adjustments to this protocol may be applied; in example, conjugated secondary antibody may be chosen for specificity or non-cross-reactivity.

10   E.   Cell fusion: The animal selected for hybridoma production is intravenously injected with 50 to 100 μg of ag in PBS. Four days later, the animal is sacrificed by carbon dioxide and its spleen collected under sterile conditions into 35 ml Dulbeccos' Modified

15   Eagle's Medium containing 200 U/ml Penicillin G, 200 μg/ml Streptomycin Sulfate, and 4 mM glutamine (2x P/S/G DMEM). The spleen is trimmed of excess fatty tissue, then rinsed through 4 dishes of clean 2x P/S/G DMEM. It is next transferred to a sterile stomacher

20   bag (Tekmar, Cincinnati, OH) containing 10 ml of 2x P/S/G DMEM and disrupted to single cell suspension with the Stomacher Lab Blender 80 (Seward Laboratory UAC House; London, England). As cells are released from the spleen capsule into the media, they are removed

25   from the bag and transferred to a sterile 50 ml conical centrifuge tube (Becton Dickinson and Company, Lincoln Park, NJ). Fresh media is added to the bag and the process is continued until the entire cell content of the spleen is released. These splenocytes are washed 3

30   times by centrifugation at 225 x g for 10 minutes.

        Concurrently, log phase cultures of myeloma cells, Sp2/0-Ag14 or Y3-Ag1.2.3 for mouse or rat splenocyte fusions, respectively, (American Type Culture Collection; Rockville, MD) grown in complete

35   medium (DMEM, 10% inactivated fetal bovine serum, 2 mM

glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10 mM hepes buffer; Gibco Laboratories, Grand Island, NY) are washed in similar fashion. The splenocytes are combined with the myeloma cells and pelleted once again. The media is aspirated from the cell pellet and 2 ml of polyethylene glycol 1500 (PEG 1500; Boehringer Mannheim Biochemicals, Indianapolis, IN) is gently mixed into the cells over the course of 1 minute. Thereafter, an equal volume of 2x P/S/G DMEM is slowly added. The cells are allowed to fuse at 37° C for 2 minutes, then an additional 6 ml of 2x P/S/G DMEM is added. The cells are again set at 37°C for 3 minutes. Finally, 35 ml of 2x P/S/G DMEM is added to the cell suspension, and the cells pelleted by centrifugation. Media is aspirated from the pellet and the cells gently resuspended in complete medium. The cells are distributed over 96-well flat-bottom tissue culture plates (Becton Dickinson Labware; Lincoln Park, NJ) by single drops from a 5 ml pipette. Plates are incubated overnight in humidified conditions at 37°C , 5% CO_2. The next day, an equal volume of selection medium is added to each well. Selection consists of 0.1 mM hypoxanthine, 4×10^{-4} mM aminopterin, and 1.6×10^{-2} mM thymidine in complete medium. The fusion plates are incubated for 7 days followed by 2 changes of medium during the next 3 days; HAT selection medium is used after each fluid change. Tissue culture supernatants are taken 3 to 4 days after the last fluid change from each hybrid-containing well and tested by EIA for specific antibody reactivity. This protocol has been modified by that in Hudson and Hay, "Practical Immunology, Second Edition", Blackwell Scientific Publications.

Example 12

Cloning of an OPG Binding Protein Receptor
Expressed on Hematopoietic Precursor cells

Biologically active recombinant murine OPG
5 binding protein [158-316] was conjugated to
fluorescein-isothyocyanate (FITC) to generate a
fluorescent probe. Fluorescent labeling was performed
by incubation of recombinant murine OPG binding protein
[158-316] with 6-fluorescein-5-(and 6) carboxyamido
10 hexanoic acid succinimidyl ester (Molecular Probes,
Eugene, OR) at a 1:6 molar ratio for 12 hrs. at 4°C.
FITC-labeled OPG binding protein [158-316] was further
purified by gel filtration chromatography. Mouse bone
marrow cells were isolated and incubated in culture in
15 the presence of CSF-1 and OPG binding protein [158-316]
as described in Example 10. Mouse bone marrow cells
were cultured overnight in CSF-1 (30 ng/ml) and OPG
binding protein [158-316] (20 ng/ml). Non-adherent
cells were removed first and stored on ice and the
20 remaining adherent cells were removed by incubating
with cell dissociation buffer (Sigma Chemicals, St.
Louis, MO), pooled with the non-adherent population,
and then stained with FITC-OPG binding protein as
described above. After washing and resuspending in PBS
25 with 0.5% BSA, the cells were exposed to FITC-OPG
binding protein, washed, then sorted by FACS. The
population of cells that were positive for staining
with the FITC-OPG binding protein was collected and
mRNA was isolated as described in Example 2. This mRNA
30 preparation was used to make a cDNA library following
procedures described in Example 2.
        The cDNA library produced from this source
was used for random EST sequence analysis as previously
described in PCT Publication No. WO97/23614 and in
35 Simonet et al. (Cell 89, 309-319 (1997)). Using this

method, an ~2.1 kb cDNA was detected that encoded a
novel TNFR-related protein. The long open reading
frame of the murine ODAR cDNA encodes a protein of 625
amino acid residues and contains the hallmark features

5   of TNFR-related proteins: a hydrophobic signal peptide
at its N-termini, four tandem cysteine-rich repeat
sequences, a hydrophobic transmembrane domain, and a
cytoplasmic signaling domain. The homology of this
protein with other members of the TNF receptor family

10   and its expression in bone marrow cells that bind FITC-
labeled OPG binding protein suggest that it is a
potential receptor for the TNF-related OPG binding
protein. This protein is designated ODAR, or
osteoclast differentiation and activation receptor.

15   The nucleic acid sequence and predicted amino acid
sequence of murine ODAR is shown in Figure 10.

　　　　Recent analysis of sequences in publicly
available databases indicate that this protein is the
murine homolog of a human TNFR-related protein known as

20   RANK (Anderson et al., Nature $\underline{390}$, 175-179 (1997)).

<u>Example 13</u>
Production of Recombinant ODAR Protein in Mammalian
Cells

25

　　　　A soluble ODAR extracellular domain fused to
the Fc region of human IgG_1 was produced using
procedures for the construction and expression of Fc
fusion proteins as previously described in WO97/23614

30   and in Simonet et al., <u>supra</u>. To generate soluble ODAR
protein in mammalian cells, cDNA encoding extracellular
domain of murine ODAR (amino acids 27-211) was PCR
amplified with the following set of oligonucleotide
primers:

35

5' TCT CCA AGC TTG TGA CTC TCC AGG TCA CTC C-3'
   (SEQ ID NO: 37)

5' TCT CCG CGG CCG CGT AAG CCT GGG CCT CAT TGG GTG-3'
   (SEQ ID NO: 38)

PCR reactions were carried in a volume of 50 µl with 1 unit of vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X100, 10 µM of each dNTP, 1µM of each primer and 10 ng of ODAR cDNA template. Reactions were performed in 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, for a total of 16 cycles. The PCR fragment was isolated by electrophoresis. The PCR fragment creates a Hind III restriction site at 5' end and a Not I restriction site at 3' end. The Hind III-Not I digested PCR fragment was then subcloned in-frame into a modified pCEP4-Fc vector in front of the human IgG-γ1 heavy chain sequence as described previously in WO97/23614 and in Simonet et al. supra). A linker was introduced which encodes two irrelevant amino acids spanning the junction between the ODAR extracellular domain and the IgG Fc region.

The construct was then digested with Nhe I and Hind III and the following annealed oligonucleotide pair encoding OPG signal peptide (amino acid 1-21) was inserted in-frame:

5'CTA GCA CCA TGA ACA AGT GGC TGT GCT GCG CAC TCC TGG TGC TCC TGG ACA TCA TTG AAT GGA CAA CCC AGA-3'   (SEQ ID NO: 39)

5'AGC TTC TGG GTT GTC CAT TCA ATG ATG TCC AGG AGC ACC AGG AGT GCG CAG CAC AGC CAC TTG TTC ATG GTG-3'   (SEQ ID NO: 40)

A linker which encodes two irrelevant amino acids was introduced between OPG signal peptide and ODAR sequences. The final engineered construct (ODAR-Fc/pCEP4) encodes a fusion protein containing from

5   amino terminus to carboxy terminus: OPG signal peptide (amino acids 1-21)-linker (LysLeu)-ODAR (amino acids 27-211)-linker (AlaAla)-human IgG Fc.

     The construct was transfected into 293-EBNA-1

10   cells by calcium phosphate method as described (Ausubel et al., Curr. Prot. Mol. Biol. 1, 9.1.1-9.1.3, (1994). The transfected cells were then selected in 200 µg/ml hygromycin (GibcoBRL) and the resulting drug-resistant mass cultures were pooled and grown to confluence. The

15   cells were washed in PBS once and then cultured in serum-free media for 72 hr. The conditioned media was collected. The ODAR-Fc fusion protein in the media was detected by western blot analysis with anti-human IgG Fc antibody.

20   The Fc fusion protein was purified by protein-A column chromatography (Pierce) using the manufacturer's recommended procedures. Fifty pmoles of the purified protein was then subjected to N-terminal sequence analysis by automated Edman degradation as

25   essentially described by Matsudaira et al (J. Biol. Chem. <u>262</u>, 10-35 (1987)). The following amino acid sequence was read after 10 cycles:

       NH_2- K L V T L Q V T P-CO_2H.

30   The binding activity of ODAR-Fc with OPG binding protein was examined by immunofluorescent staining of transfected COS-7 cell cultures as described in Example 2. COS-7 cells were lipofected with 1µg of an expression vector containing DNA

35   encoding murine OPG binding protein. After 48 hr

incubation, cells were then incubated in PBS-FBS
solution containing 10 mg/μl of human IgG Fc, ODAR-Fc,
or OPG-Fc protein at 4°C for 1 hr. Cells were then
washed with PBS twice and then incubated in PBS-FBS
5   solution containing 20 μg/ml FITC-labeled goat anti-
human IgG (Southern Biotech Associates) for another hr.
After washing with PBS, cells were examined by confocal
microscopy (ACAS, Ultima, Insight Biomedical Imaging,
Inc., Okemos, MI). Both ODAR-Fc and OPG-Fc bind to
10   OPGL transfected COS-7 cells (Figure 11).

Example 14
In vitro biological activity of recombinant soluble
ODAR

15

The ability of ODAR to inhibit stimulation of
osteoclast formation by OPG binding protein was
assessed in a mouse bone marrow culture in the presence
of CSF-1 (30ng/ml) and OPG binding protein (5ng/ml).
20   Procedures for the use of mouse bone marrow cultures to
study osteoclast maturation are described in WO97/23614
and in Example 8. ODAR-Fc fusion protein produced as
described in Example 12 was added to concentrations of
65 to 1500ng/ml. Osteoclast formation was assessed by
25   tartrate resistant alkaline phosphotase (TRAP)
cytochemistry and the TRAP solution assay after five
days in culture.
A dose dependent inhibition of osteoclast
formation by ODAR-Fc fusion was observed both by
30   cytochemistry and by TRAP activity (Figure 12). ODAR-
Fc fusion protein inhibited osteoclast formation with
an ED_{50} of about 10-50ng/ml.

Example 15
35   In vivo biological activity of recombinant soluble ODAR

Young rapidly growing male BDF1 mice aged 3-4
weeks received varying doses of ODAR-Fc fusion protein
by single daily subcutaneous injection in carrier
5   (PBS/0.1% BSA) for four days. The mice were x-rayed on
day 5. Doses of ODAR-Fc fusion protein used were 0.5,
1.5 and 5mg/kg/day. For each treatment, all the mice
in that group and in the control group that received
PBS/0.1% BSA were x-rayed on a single film. The
10   proximal tibial metaphyseal region was compared between
pairs of control and treated tibias and scored as a "+"
if the treated tibia was denser by visual assessment
than the control giving the 8 scores shown below. An
arbitrary score of 5/8 was required for a "positive"
15   result. (Dose is in mg/Kg/day). (n=4).

After sacrifice the right tibia was removed
from each animal and the bone density in the proximal
tibial metaphysis was measured by peripheral
quantitative computerized tomography (pQCT) (Stratec,
20   Germany). Two 0.5 mm cross-sections of bone, 1.5 mm and
2.0 mm from the proximal end of the tibia were analyzed
(XMICE 5.2, Stratec, Germany) to determine total bone
mineral density in the metaphysis. A soft tissue
separation threshold of 1500 was used to define the
25   boundary of the metaphyseal bone.

ODAR-Fc administration in young growing mice
inhibited bone resorption at the proximal tibial growth
plate producing a region of increased bone density that
was evident visually on radiographs. Radiographic
30   changes were apparent at a dose of 1.5mg/kg/day and
above in two experiments (Table 1). Measurement of the
bone density by pQCT in samples from the second
experiment in a similar region of the tibia confirmed
the dose dependent increased in bone density in these
35   mice (Figure 13).

Table 1

Inhibition of bone resorption by ODAR-Fc fusion protein

Experiment #1

Factor	Dose	1	2	3	4	5	6	7	8	Result
ODAR-Fc·	5.0	+	+	+	+	+	+	+	+	Positive 8/8
ODAR-Fc	1.5	–	+	+	–	+	+	+	+	Positive 6/8
ODAR-Fc	0.5	–	–	–	–	–	–	–	–	Negative 0/8
ODAR-Fc	0.15	–	–	–	–	–	–	–	–	Negative 0/8

Experiment #2

Factor	Dose	1	2	3	4	5	6	7	8	Result
ODAR-Fc	5.0	+	+	+	+	+	+	+	+	Positive 8/8
ODAR-Fc	1.5	+	+	+	+	+	+	+	+	Positive 8/8
ODAR-Fc	0.5	–	–	–	+	–	–	–	–	Negative 1/8

*   *   *

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     While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.